Chinese Science Bulletin 2004 Vol. 49 No. 24 2620—2626

Stable carbon and hydrogen isotopic fractionations of alkane compounds and crude oil during aerobically microbial degradation

PENG Xianzhi, ZHANG Gan, CHEN Fanzhong & LIU Guoqing

State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
Correspondence should be addressed to Peng Xianzhi (e-mail: pengx@gig.ac.cn)

Abstract **Normal alkane compounds dodecane, pentadecane, hexadecane, octadecane, tetracosane, isoprenoid alkane pristane and a crude oil sample were aerobically biodegraded with a pure bacterial strain GIM2.5 and white rot fungus *Phanerochaete Chrysosporium*-1767 to monitor the kinetic fractionation of the molecular stable carbon ($d^{13}C$) and hydrogen (d D) isotopes in the course of biodegradation. Both $d^{13}C$ (V-PDB) and d D (V-SMOW) remained stable for the standard alkane compounds and *n*-alkane components (from *n*-C_{13} to *n*-C_{25}) of the crude oil, generally varying in the range of ±0.5‰ and ±5‰, respectively, within the range of the instrumental precisions, especially for those molecularly heavier than *n*-C_{16} during microbial degradation. These results indicate that molecular stable carbon and hydrogen isotopic fingerprints can be promising indicators for tracing the sources of petroleum-related contaminants in the environment, especially in the case of severe weathering when they are difficult to be unambiguously identified by the chemical fingerprints alone.**

Keywords: aerobic biodegradation, alkanes, stable isotopic fingerprinting, environmental indicator, petroleum-related contamination.

DOI: 10.1360/03wd0281

Petroleum-related environmental pollution has become a global concern with the industrial and economical development, causing serious ecological risks and threatening natural resources and human health. Therefore, it is important to characterize contaminants and thereby identify unambiguously the sources of contaminants, not only for the purpose of settling disputes related to liability but more importantly, for selecting appropriate responses and taking effective cleanup precautions[1].

Chemical fingerprints obtained from advanced instrumental analysis such as GC-FID and GC-MS have long been used effectively to characterize crude oils and their refined products[1]. However, the oils can be altered drastically as the result of weathering processes in the environment, such as evaporation, dissolution, biotic and other abiotic degradation, making it difficult to correlate the contaminants with the source oils. In addition, the lack of specificity of chromatographic fingerprints also makes it difficult to differentiate among possible source oils simply by using chemical profiles. Hence, molecular stable isotopic fingerprinting that depends on the origin and the environmental fate can be used as a prominent alternative or complimentary tool in the source identification of petroleum-related contamination due to their specificity and environmental stability[2−5].

Several studies have been reported recently on the potential application of stable isotopic fingerprints as environmental indicators of pathogenic contamination. For example, $d^{13}C$ measurements of chlorinated ethenes and gasoline additives including BETX and MTBE were widely used to identify the gasoline pollution in groundwater[6−9]. This approach has also been used in PAHs source tracing and apportionment[10−12] as well as in biodegradation monitoring[13−16]. Mansuy et al. (1997) identified a weathered oil spill by use of the molecular stable carbon isotopic composition of saturated hydrocarbons. It appears therefore that molecular stable isotopic fingerprinting is helpful in source differentiation and for the environmental fate monitoring as well as natural attenuation of petroleum hydrocarbon pollutants, particularly for samples where molecular biomarkers have been removed as the result of extensive weathering. Recently, stable carbon isotope fractionation was even applied to quantifying bioremediation of individual compounds[16] and to distinguishing aerobic and anaerobic degradation of chlorinated ethane in field applications of *in situ* biodegradation[17].

However, the kinetically isotopic fractionation in the course of weathering processes is essential for the application of isotopic fingerprinting as an alternative of environmental indicators. It was reported that no obvious fractionation of stable carbon isotopic compositions for *n*-alkanes and PAHs occurred during vaporization, adsorption[2,9] and aerobic biodegradation[18]. On the other hand, there were also reports that $d^{13}C$ and d D values became enriched during bacterial biodegradation[7,8,19]. d D enrichment was observed for C_{15} to C_{18} *n*-alkanes but not for those with longer chains (*n*-C_{19} to *n*-C_{27}) during aerobic biodegradation[20], and large d D enrichment was reported[4,21] for toluene, MTBE and BTEX as the results of both anaerobic and aerobic biodegradation.

In this study, we focus attention on the possible kinetic stable carbon and hydrogen isotopic fractionation of *n*-alkanes induced by aerobic microbial degradation, which is an important environmental process, and the effect of microorganism species on isotopic fractionations. Standard *n*-dodecane, *n*-pentadecane, *n*-hexadecane, *n*-octadecane, *n*-tetracosane and isoprenoid alkane pristane, common components of crude oils and the refined products, and a crude oil sample were microbiologically

degraded with a pure bacterial strain (GIM2.5) and a white rot fungus (*Phanerochaete Chrysosporium*-1767) to see if isotopic fractionation occurred during aerobic biodegradation by these microorganism species.

1 Methodology and experiments

(i) Chemicals and reagents. Standard *n*-dodecane and *n*-hexadecane (99.0%—99.5% of purity) were products of the Tianjin Chemical Reagent Company, China; *n*-pentadecane was bought from Fisher Scientific Worldwide Company, HK and *n*-octadecane, *n*-tetracosane and isoprenoid pristane were purchased from Chem Service, with purity of 98.9% and 99% respectively. Standard *n*-alkane mixtures (*n*-C_{12} to *n*-C_{32}) with known $d^{13}C$ and dD values were derived from Indiana University, USA. Deuterated *n*-eicosane ($C_{20}D_{42}$) was bought from Sigma, USA. Methylene chloride (DCM), acetone, methanol and *n*-hexane were re-distilled. Alumina and silica gel (100—200 mesh) were Soxhlet extracted with a mixture of methylene chloride and methanol for 72 h, followed by activation at 250 and 180℃ overnight respectively and then deactivation with 3% distilled water. Urea was washed with DCM and then air-dried in a fumehood. Anhydrous sodium sulfate was baked at 500℃ for 24 h before use. The oil sample studied was provided by Prof. Fan Shanfa in Guangzhou Institute of Geochemistry, Chinese Academy of Sciences.

The pure bacterial strain GIM2.5, isolated from petroleum contaminated sediment, was provided by Dr. Sun Guoping in Guangzhou Institute of Microbiology, Guangdong Academy of Sciences. The bacteria was identified to be *Pseudomonas alcaligenes Monias*, showing negative Gram reaction. The white rot fungus *Phanerochaete Chrysosporium*-1767 was provided by Dr. Fu Shiyu in Guangzhou Institute of Chemistry, Chinese Academy of Sciences.

(ii) Microorganism pre-culturing. The mineral medium consisted of (per liter of water) K_2HPO_4, 1 g; KH_2PO_4, 1 g; NH_4NO_3, 1 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 5 g; $CaCl_2$, 0.002 g; trace $CuSO_4$, $ZnSO_4$ and $FeSO_4$ (about 0.001 g). The pH was adjusted to 6.8 with 0.5 mol/L HCl and 1 mol/L NaOH. Normal alkanes and oil were sterilized with 0.25 μm filters before use.

Bacteria GIM2.5 was inoculated into 100 mL autoclaved mineral medium (in a 250 mL flask) containing 1.5 mg of *n*-dodecane, 1.5 mg of *n*-pentadecane, 1.5 mg of *n*-octadecane, 1.5 mg of *n*-tetracosane, 0.8 mg of *n*-hexadecane and 2 μL of pristane and 0.55 mmol/L glucose (filter sterilized) as carbon and energy source. The incubation was carried out in a vibrator at room temperature (RT, 70 r/min) for 18 h, when the bacteria growth was obviously observed. The bacteria was collected and preserved in NaCl buffer at 4℃ before use in 1 week.

Sterilized crude oil (5 μL) was added into 100 mL autoclaved mineral medium containing 2 g/L glucose in a 250 mL flask before inoculation of the white rot fungus *Phanerochaete Chrysosporium*-1767. The fungus was harvested after being incubated in a temperature-constant vibrator at 39℃ (70 r/min) for 48 h and preserved in NaCl buffer at 4℃ for use in a week.

(iii) Biodegradation experiments. Five microlitres of suspended solution of bacteria GIM2.5 were inoculated in 250 mL flasks each containing 125 mL of autoclaved mineral media supplemented with 12.5 mg of *n*-dodecane, 12.5 mg of *n*-pentadecane, 12.5 mg of *n*-hexadecane, 6.25 mg of *n*-octadecane, 6.25 mg of *n*-tetracosane, and 5 μL of pristine as carbon and energy source. The biodegradation was carried out in a temperature-constant vibrator at 30℃ (90 r/min). Samples were collected after 0, 6, 12, 24, 48, 57, 72, 96, 120, 144, 168, 192 and 240 h. Blank (uninoculated) and control (uninoculated plus HgCl as biocide) flasks were also set up at 6, 12, 24, 48, 57 and 96 h to check any possible isotopic fractionation and loss of alkanes that may be caused by abiotic processes such as leakage and evaporation and the possibility of any interruption from airborn microbial activities during the experiments. All assays were performed in triplicate. The biodegradation samples, blanks and controls were treated in the same manner before analyzing residue concentrations and isotopic compositions.

The suspension of pre-cultured *Phanerochaete Chrysosporium*-1767 was inoculated in 500 mL of autoclaved mineral media after adding 1 g/L glucose and 50 μL of crude oil as carbon and energy source. Biodegradation of crude oil was aerobically carried out in a temperature-constant vibrator (39℃ and 90 r/min). Samples were collected after 0, 24, 72, 120, 168, 240 and 336 h.

(iv) Extraction. Samples were centrifuged at 4000 r/min for 10 min. The supernatants were extracted 3 times with 20 mL re-distilled methylene chloride. The inner wall of centrifuge tubes were also wiped with precleaned absorbent cotton after removing the microorganism to collect possible adherent targets. The cotton was eluted with DCM. The extracts were then combined and dehydrated with baked anhydrous sodium sulfate and reduced to small volumes by rotary evaporation at 28℃. The solvent was then transferred to *n*-hexane and concentrated to a final volume of 1 mL under a gentle nitrogen flow.

The crude oil samples were fractionated by 9 mm (i.d.)×400 mm (length) grass columns packed with mixtures of deactivated silica gel and alumina (2:1, volume ratio). Hexane (30 mL) was used to elute the saturated components. The eluates were further cleaned by urea adduction to remove branched and cyclic components before isotopic analysis.

(v) GC-FID analysis. Samples were analyzed by GC (HP 6890)-FID with split injection (1:2) after adding $C_{20}D_{42}$ (2.196 μg) as internal standard to monitor the bio-

degradation of alkanes and oil. The injector temperature was maintained at 290℃. A DB-5 (30 m × 0.32 mm i.d. × 0.25 μm film thickness) capillary column was used. The GC oven temperature was programmed as follows: from 40℃ (2 min) ramp to 300℃ at the rate of 4℃/min, with a final holding for 30 min. The carrier gas was highly pure nitrogen.

(vi) Stable isotopic composition analysis. Stable carbon and hydrogen compositions were determined using a GC (HP 6890) coupled with a Finnigan MAT Delta Plus XL isotopic ratio MS (IRMS) and an on-column injector. A DB-5 (30 m × 0.32 mm i.d. × 0.25 μm film thickness) capillary column was used. GC oven temperature program for the standard n-alkanes and pristane was: 50—120℃ (3 min.) at 15℃/min, to 220℃ at 5℃/min and then to 300℃ (5 min) at 20℃/min. For crude oil alkane components, the program was: 50—170℃ at 15℃/min, to 220 at 5℃/min, and to 300℃ (10 min) at 20℃/min. Helium was used as the carrier gas. d^{13}C and dD were reported relative to PDB and SMOW standards respectively.

CO_2 and H_2 reference gases were automatically introduced into the IRMS in six pulses at the beginning and end of each analysis of d^{13}C and dD respectively for the purpose of standardization. Reproducibility and accuracy were verified daily by injection of standard n-alkanes with known d^{13}C and dD (from Indiana University), which are in the range of 0.5‰ and 5‰ for d^{13}C and dD respectively. The H_3 factor was determined using the standard reference H_2 gas introduced into the IRMS to ensure a daily variability of ≤0.1. At least triplicate injections were performed for each sample, and the results presented in this paper were geometric averages of triplicate injections. Generally, both d^{13}C and dD values based on the peak heights in V of mass 1.5—5 signals were adopted.

2 Results and discussion

(i) Biodegradation of standard *n*-alkanes and crude oil. The mechanisms of biodegradation of alkanes and the intermediate products had been comprehensively studied[22,23]. So, we will not discuss them here.

The growth of bacterium GIM2.5 with the mixture of alkanes as sole carbon and energy source during biodegradation was monitored by measuring the optical density using a spectrophotometer at 550 nm wavelength (OD_{550}, Fig. 1). GIM2.5 grew quickly to exponential phase in 72 h. During this time, the *n*-alkanes were rapidly degraded by more than 90% for *n*-dodecane, 82% for *n*-pentadecane and 78% for *n*-hexadecane, whereas only 30% of pristane was degraded. However, the biodegradation then became slower. Only traces *n*-dodecane was detected after 144 h, and about 98% of *n*-pentadecane and *n*-hexadecane had been degraded after 192 h. In contrast, more than 40% and 90% were detected for *n*-dodecane and *n*-pentadecane and *n*-hexadecane respectively in blank and control flasks after 96 h (Fig. 1). The relative large loss of dodecane was possibly attributable to the significant volatilization during the shaking.

The saturated components of the crude oil were in the range of *n*-C_9 to *n*-C_{33}, with the highest peaks at *n*-C_{13} to *n*-C_{15}. The pattern evolution of the saturated fraction of the crude oil during biodegradation by the fungus *Phanerochaete Chrysosporium*-1767 is shown in Fig. 2. The lighter components were degraded faster, resulting in the relative increase of proportion of the heavier components. The heavier alkanes became dominant and *n*-C_{25} peaked after 14 d of biodegradation. The ratios of *n*-C_{17}/pristane and *n*-C_{18}/phytane have been used to evaluate the biodegradation of crude oils[24]. In our experiments, the ratios of *n*-C_{17}/pristane and *n*-C_{18}/phytane of the original crude oil sample were 29 and 23 respectively; they decreased to 4.0 and 3.1 after 8 d and 2.9 and 2.2 after 14 d of biodegradation by *Phanerochaete Chrysosporium-1767*.

(ii) Stable carbon isotopic fractionation of alkane standards during biodegradation. The stable carbon isotopic compositions of the residual *n*-alkane and pristane standards in the course of aerobic biodegradation by bacterium GIM2.5 are shown in Table 1. The d^{13}C values of *n*-dodecane, *n*-pentadecane, and *n*-hexadecane varied in the range of −28.87‰—−28.26‰, −30.11‰—−29.53‰, −34.34‰—−33.78‰ and −32.15‰—−32.3‰ respectively during 240 h of biodegradation. The above isotopic fractionation was in the range of instrument accuracy (0.5‰). As for pristane, larger enrichment (1.63‰ and 1.57‰) at 144 and 168 h was possibly related with significant deviation caused by lower instrumental mass signals (<0.8 V) because of the low concentration. We had limited data for that of *n*-tetracosane. However, the isotopic composition of *n*-tetracosane remained considerably stable from 144 to 240 h for microbial degradation. This result showed that little stable carbon isotopic fractionation occurred as the result of the aerobic biodegradation by bacterium GIM2.5 for middle to long chain alkanes, which are common components of crude oils and their refined products.

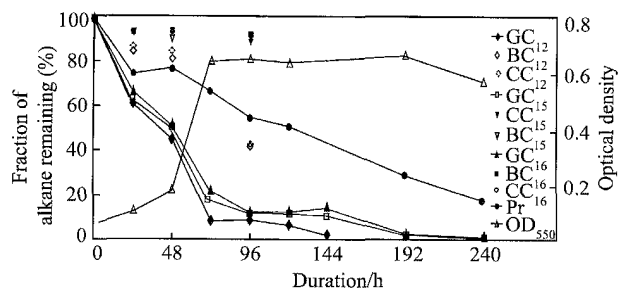

Fig. 1. Microbial degradation of alkane compounds by pure bacterium strain GIM2.5. GC_i, biodegradation samples; BC_i, blank samples; CC_i, control samples, Pr, pristane.

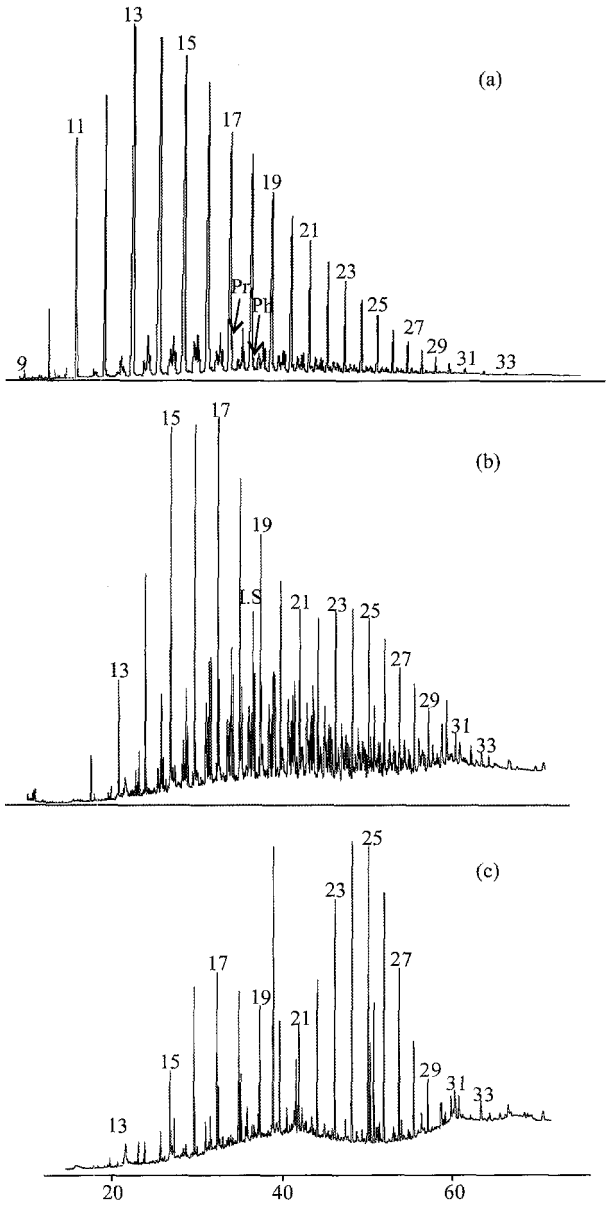

Fig. 2. Evolution of saturated components of the oil sample in the course of biodegradation by white rot Fungus Phanerochaete Chrysosporium-1767. (a) Original oil; (b) biodegradation of 8 d; (c) biodegradation of 14 d. I.S, internal standard; Pr, pristane; Ph, phytane. Numbers above peaks indicate chain length of normal alkanes.

(**iii**) Evolution of stable hydrogen isotopic composition of alkane standards during aerobic biodegradation. Table 2 shows the stable hydrogen isotopic compositions (dD) of residual alkane standards during aerobic biodegradation by bacterium GIM 2.5 as well for the blank and control samples. Over 48 h, when 45% of *n*-dodecane was degraded, its dD value varied from −146.96‰ to −152.14‰ comparable with the results from control and blank experiments. Even though >85% *n*-pentadecane and *n*-hexadecane were degraded after 192 h, the dD values remained relatively constant (−157.70‰ — −148.54‰ and −129.93‰ — −123.52‰, respectively). dD of pristane, *n*-octadecane and *n*-tetracosane were in the range of −365.99‰ — −363.80‰, −127.75‰ — −135.63‰ and −38.17‰ — −35.38‰, respectively in the course of biodegradation, within the scope of instrumental sensitivity and accuracy. No difference of dD alterations was found between the biodegraded samples from the blank and control samples in the experiments. However, we also found little dD enrichment for *n*-pentadecane in the course of biodegradation (ΔdD in the range of –2.51‰ and 8.81‰), but somewhat dD depletion for *n*-dodecane after 72 h of degradation (ΔdD in the range of −14.42‰ and 3.31‰). It was reported by Wang and Huang[25] that dD values for short-chain alkanes were depleted during the course of volatilization. However, if the dD depletion of *n*-dodecane is relevant to the volatilization during the shaking in the vibrator is still pending.

(**iv**) Stable hydrogen isotopic fractionation of saturated components of crude oil during aerobic degradation. No significant fractionation of the stable hydrogen isotopic compositions occurred for the saturated components from *n*-C_{13} to *n*-C_{25} of the crude oil in the course of biodegradation by the fungus *Phanerochaete Chrysosporium*-1767 (Fig. 3). Variations of dD values were in the range of −10‰ — 2.56‰ (within the 2s of the analytical uncertainties for hydrogen isotopic measurements), whereas dD values for alkanes heavier than n-hexadecane remain constant during microbial degradation.

A prerequisite for the application of isotopic fingerprinting as an environmental source tracer relies on the isotopic stability during environmental weathering processes, particularly in the course of biodegradation. A comprehensive study by Morasch et al.[26] elucidated that isotopic fractionation depends on enzymatic pathways involved rather than on the simple mass transform of compounds caused by metabolism. It implies from the previous studies that different microbial communities or environmental conditions may play controls on whether or not significant fractionation is associated with biodegradation of hydrocarbons[7,17,20]. Based on our work and others', isotopic compositions tend to be stable or fractionation is significantly smaller under aerobic biodegradation compared to those by anaerobic biodegradation, especially those of larger molecular weight compounds.

In our work, we used middle to long chain alkanes (*n*-C_{12} to *n*-C_{25}) to see the isotopic fractionation in the course of aerobically microbial degradation. No obvious fractionation was observed for both stable carbon and hydrogen isotopes of these compounds. However, Pond et al. (2002) reported that 12‰–25‰ enrichment in dD value

Table 1 Evolvement of stable carbon isotopic compositions of standard *n*-alkanes and pristane during aerobic degradation by GIM2.5 ($d^{13}C$, ‰V- PDB)[a)]

Duration/h	*n*-C_{12}		*n*-C_{15}		*n*-C_{16}		Pristane		*n*-C_{24}
	f (%)	$d^{13}C$	*f* (%)	$d^{13}C$	*f* (%)	$d^{13}C$	*f* (%)	$d^{13}C$	$d^{13}C$
0	100	–28.72	100	–30.03	100	–34.34	100	–26.77	na
24	61	–28.87	64	–30.11	67	–34.24	75	–26.83	na
48	45	–28.56	51	–29.86	52	–34.21	77	–26.80	na
72	9	–28.26	18	–29.85	22	–33.92	67	–26.86	na
96	9	–28.50	13	–29.86	13	–34.05	55	–26.84	na
120	7	–28.38	12	–29.97	13	–34.06	51	–26.79	na
144	1	nd	11	–29.64	15.4	–33.78	na	25.14[b)]	–31.89
168	na	nd	na	–29.61	na	–34.02	29	25.20[b)]	na
192	0.5	–28.08	1.6	–29.59	2.2	–33.98	na	nd	–32.15
240	0	nd	0.3	–29.53	0.4	–33.80	17.6	nd	–32.3
$\Delta d^{13}C$[4]	–0.85—0.1		–0.08—0.5		0.1—0.54		–0.09—1.63		

a) means of triplicate analyses, 1ó <0.5‰; *f*, remaining fraction of the alkane standards; nd, too trace to measure; na, not analyzed; $\Delta d^{13}C = d^{13}C(t) - d^{13}C(0)$, where $d^{13}C_{12}(0)$ is the $d^{13}C$ value of $t = 0$. b) Mass signal < 0.8 V, resulting in large deviation in $d^{13}C_{12}$ values.

Table 2 Stable hydrogen compositions of residue standard *n*-alkanes and pristane in the course of biodegradation by GIM2.5 (d D, ‰V-SMOW)[a)]

Dura-tion/h	*n*-C_{12}			GC_{15}	GC_{16}	GPristane	*n*-C_{18}			*n*-C_{24}		
	GC_{12}	BC_{12}	CC_{12}				GC_{18}	BC_{18}	CC_{18}	GC_{24}	BC_{24}	CC_{24}
Original	–151.19	–151.19	–151.19	–157.35	–128.39	–363.78	–132.96	–132.96	–132.96	–35.57	–35.57	–35.57
6	–146.96	na	–153.70	nd[6]	nd	Nd	–131.59	na	–132.90	–36.52	na	–40.60
12	–148.58	na	–149.71	nd	nd	Nd	–134.00	na	–129.42	–36.84	na	–38.58
24	–152.14	–149.12	–150.88	–153.81	–126.99	Nd	–135.63	–132.45	–133.15	–36.89	–39.30	–35.53
48	–148.08	na	na	–151.78	–129.93	Nd	nd	na	na	nd	na	na
57	nd	–155.31	–145.21	nd	nd	Nd	–128.42	–128.21	–130.82	–38.17	–41.05	–37.50
72	–165.61	na	na	–157.70	–126.68	–363.80	nd	na	na	nd	na	na
96	–160.73	–152.89	–147.41	–153.58	–125.99	–365.38	–134.87	–129.12	–128.32	nd	–38.92	–36.65
120	–160.53	na	na	–156.36	–133.73	–363.91	nd	na	na	nd	na	na
144	–158.46	na	na	–148.54	–123.52	Nd	–128.44	na	na	–35.38	na	na
168	–159.70	na	na	–153.00	–124.49	Nd	–127.75	na	na	–37.65	na	na
192	nd	na	na	–161.41	–126.77	–364.68	nd	na	na	–41.87	na	na
240	nd	na	na	nd	Nd	–365.99	nd	na	na	nd	na	na
Δd D (‰)	–14.42—3.11	–4.12—2.07	–2.51—5.98	–2.51—8.81	–5.34—–4.87	0.02—2.21	–1.91—5.21	0.51—4.75	–0.19–4.64	–6.3—–0.19	–5.48—–3.35	–5.03—0.04

a) Mean values of triplicate analysis, 1 *s* <5 ‰, GC_i, microbial degradation alkane samples; BC_i, blank sample; CC_i, control samples; nd, too trace to measure; na, not analyzed; Δd D = d D(t)–d D(0), where d D(t) is the hydrogen isotopic value of alkanes at time t, and d D(0) is the original hydrogen isotopic value of alkanes.

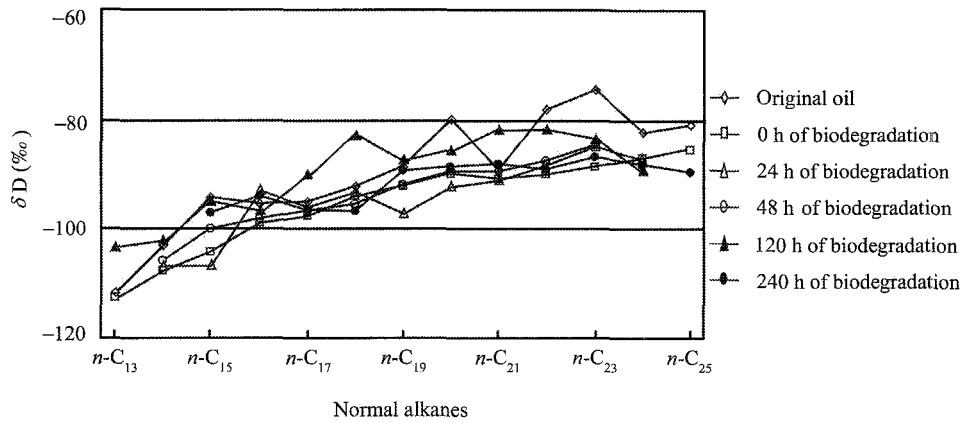

Fig. 3. Stable hydrogen isotopic compositions of n-alkane components of the oil during the biodegradation by white rot fungus Phanerochaete Chrysosporium-1767.

for *n*-alkanes from *n*-C_{15} to *n*-C_{18} occurred during biodegradation by a microorganism consortium isolated from activated sludge polluted by petroleum hydrocarbons. In addition, several laboratory and field studies showed that anaerobic biodegradation produces significant isotopic enrichments for hydrocarbons such as trichloroethylene (TCE), BETX, MTBE and alkybenzenes[7,19,21]. These differences may be the results of the diversity of microorganism species under the environmental conditions as discussed above.

The alkane compounds used in our experiments are common components of crude oils and their refined products, such as petroleum and diesel oil. Most modern surface environments, such as riverine and estuarine environments are aerobic. It is shown from our work that dD and d^{13}C values of molecularly heavier *n*-alkanes, especially those heavier than *n*-hexadecane, should be reliable source tracers for petroleum contaminants in aerobic environment.

3 Summary

The application of stable isotopic fingerprinting as environmental indicator to trace sources of contaminants has incurred significant interest in the field of environmental researches. We used alkane compounds, which are common components of crude oils and their refined products, to elucidate the stable isotopic fractionation under aerobic biodegradation and the potential application in tracing petroleum-associated contaminants in environments. No significant fractionation of stable carbon and hydrogen isotopes occurred for *n*-alkanes heavier than *n*-dodecane, and isoprenoid pristane during the course of aerobic microbial degradation with pure bacterium strain GIM2.5 and the white rot fungus Phanerochaete Chrysosporium-1767. Considering that aerobic biodegradation is principally responsible for the weathering of petroleum hydrocarbons in the surface environment, molecular stable isotopic fingerprinting (dD and d^{13}C) of middle to longer chain *n*-alkanes and isoprenoid, especially those of compounds molecularly heavier than *n*-hexadecane, can be effective indicator for source identification of petroleum-related contamination. Isotopic fingerprinting is particularly useful if molecular biomarker is absent due to the environment weathering processes.

As discussed above, the kinetic fractionations of stable isotopic compositions during biodegradation are related with microorganism species as well as environmental conditions. Kinetic isotopic fractionation models should be built in order to form the basis for the application of isotopic fingerprinting for *in situ* oil spills identification and source tracing as well as fate monitoring or natural attenuation of petroleum contaminants.

Acknowledgements The authors would like to thank Prof. Shiyu Fu from Guangzhou Institute of Chemistry, Chinese Academy of Sciences and Dr. Guoping Sun from Guangzhou Institute of Microorganism for the provision of Microorganism species. Thanks to Prof. Shanfa Fan for the provision of crude oil sample. We also thank Prof. Huizhi Zhang and Dr. Chao Li for the assistance in the instrumental analysis. This work was financially supported by the National Basic Research Program of China (Grant No. 2003CB415002) and the National Natural Science Foundation of China (Grant No. 40202035), the Natural Science Foundation of Guangdong Province (021428).

References

1. Wang, Z., Fingas, M., Page, D. S., Oil spill identification, Journal of Chromatography A, 1999, 843(1-2): 369—411.
2. Mansuy, L., Philp, R. P., Allen, J., Source identification of oil spills based on the isotopic composition of individual components in weathered oil samples, Environmental Sciences and Technology, 1997, 31(12): 3417—3425.
3. Rooney, M. A., Vuletich, A. K., Griffith, C. E., Compound-specific isotope analysis as a tool for characterizing mixed oils: an example from the West of Shetlands area, Organic Geochemistry, 1998, 29(1-3): 241—259.
4. Mancini, S. A., Lacrampe-Couloume, G., Jonker, H. et al., Hydrogen isotopic enrichment: An indicator of biodegradation at a petro-

leum hydrocarbon contaminated field site, Environmental Sciences and Technology, 2002, 36(11): 2464—2470.
5. Phlip, R. P., Allen, J., Kuder, T., The use of the isotopic composition of individual compounds for correlating spilled oils and refined products in the environment with suspected sources, Environmental Forensics, 2002, 3: 341—348.
6. Kelley, C. A., Hammer, B. T, Concentration and stable isotope values of BTEX in gasoline-contaminated groundwater, Environmental Sciences and Technology, 1997, 31(9): 2469—2472.
7. Sherwood-Lollar, B., Slater, G. F., Sleep, B. et al., Stable carbon isotope evidence biodegradation of tetrachloroethene and trichloroethene at Area 6, Dover Air Force Base, Environmental Sciences and Technology, 2001, 35(2): 261—269.
8. Kolhatkar, R., Kuder, T., Philp, P. et al., Use of compound-specific stable carbon isotopic analyses to demonstrate anaerobic biodegradation of MTBE in groundwater at a gasoline release site, Environmental Sciences and Technology, 2002, 36(23): 5139—5146.
9. Smallwood, B. J., Philp, R. P., Thomas, W. B., The use of sable isotopes to differentiate specific source markers for MTBE, Environmental Forensics, 2001, 2: 215—221.
10. Okuda, T., Kumata, H., Naraoka, H. et al., Vertical distribution and $\delta^{13}C$ isotopic composition of PAHs in Chidorigafuchi Moat sediment, Japan, Organic Geochemistry, 2002, 33: 843—848.
11. Wilcke, W., Krauss, M., Amelung, W., Carbon isotope signature of polycyclic aromatic hydrocarbons (PAHs): Evidence for different sources in tropical and temperate environments, Environmental Sciences and Technology, 2002, 36(16): 3530—3535.
12. Stark, A., Abrajano, T. J., Hellou, J. et al., Molecular and isotopic characterization of polycyclic aromatic hydrocarbon distribution and sources at the international segment of the St, Lawrence River, Organic Geochemistry, 2003, 34(2): 225—237.
13. Mazeas, L., Budzinski, H., Stable carbon isotopic study ($^{12}C/^{13}C$) of the fate of petrogenic PAHs (methylphenanthrenes) during an in-situ oil spill simulation experiment, Organic Geochemistry, 2002, 33(11): 1253—1258.
14. Richnow, H. H., Annweiler, E., Michaelis, W. et al., Microbial in situ degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation, Journal of Contaminant Hydrology, 2003, 65(1): 101—120.
15. Yanik, P. J., O'Donnell, T. H., Macko, S. A. et al., The isotopic compositions of selected crude oil PAHs during biodegradation, Organic Geochemistry, 2003, 34(2): 291—304.
16. Griebler Christian, Safinowski Michael, Vieth Andrea et al., Combined application isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer, Environmental Sciences and Technology, 2004, 38(2): 617—631.
17. Chu, K. H., Mahendra, S., Song, D. L. et al., Stable carbon isotope fractionation during aerobic biodegradation of chlorinated ethenes, Environmental Sciences and Technology, 2004, 38(11): 3126—3130.
18. Mazeas, L., Budzinski, H., Raymound, N., Absence of stable isotope fractionation of saturated and polycyclic aromatic hydrocarbons during aerobic bacterial biodegradation, Organic Geochemistry, 2002, 33(11): 1259—1272.
19. Wilkes, H., Boreham, C., Harms, G. et al., Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria, Organic Geochemistry, 2000, 31(1): 101—115.
20. Pond, K. L., Huang, Y., Wang, Y. et al., Hydrogen isotopic composition of individual n-alkanes as an intrinsic tracer for bioremediation and source identification of petroleum contamination, Environmental Sciences and Technology, 2002, 36(4): 724—728.
21. Ward, J. A. M., Ahad, J. M. E., Lacrampe-Couloume, G. et al., Hydrogen isotope fractionation during methanogenic degradation of toluene: Potentail for direct verification of biodegradation, Environmental Sciences and Technology, 2000, 34(21): 4577—4581.
22. Whyte, L., Bourbonniere, L., Greer, C. W., Biodegradation of petroleum hydrocarbon by psychrotrophic pseudomonas strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways, Applied and Environmental Microbiology, 1997, 63(9): 3719—3723.
23. Whyte, L., Hawari, J., Zhou, E. et al., Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic rhodococcus sp, Applied and Environmental Microbiology, 1998, 64(7): 2578—2584.
24. Laboratory of Organic Geochemistry, the Institute of Geochemistry, Chinese Academy of Sciences, Organic Geochemistry (in Chinese), Beijing: Science Press, 1982, 91.
25. Wang, Y., Huang, Y., Hydrogen isotope fractionation of low molecular weight n-alkanes during progressive vaporization, Organic Geochemistry, 2001, 32(8): 991—998.
26. Morasch, B., Richnow, H. H., Schink, B. et al., Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspect, Applied and Environmental Microbiology, 2001, 67(10): 4842—4849.

(Received June 23, 2004; accepted July 29, 2004)